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Pharmacogenomic genotyping methodologies

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Abstract

''Personalized medicine'' based on an individual's genetic makeup is slowly becoming a reality as pharmacogenomics moves from the research setting to the clinical laboratory. Concordance studies between genotype and phenotype have shown that inherited mutations in several key drug-metabolizing enzymes, such as cytochrome P450 (*CYP*) *2D6*, *2C9*, and *2C19*, result in several distinct phenotypes that lead to different individual responses following drug administration. One of the major driving forces behind pharmacogenomics and its ability to be used effectively are the technologies that are available. A beneficial genotyping test must identify most or all of the mutations that have a significant impact on the expression or function of drug-metabolizing enzymes, transporter proteins, and/or drug receptors. Selection of the appropriate technology will be based on several issues, including prior knowledge of the mutation/polymorphism, sensitivity/specificity, sample requirements, and cost. Since the future volume of pharmacogenomic testing is anticipated to be large, automation of pharmacogenomics will also become increasingly important. This paper provides an overview of current technologies available for assessing polymorphisms on a small- to large-scale basis.

Keywords: genotyping; pharmacogenomics; technologies.

Introduction

Molecular techniques are becoming increasingly important in the diagnostic and clinical laboratory, and are starting to become a part of the medical evaluation for selecting specific pharmaceutical therapies. One of the major driving forces behind pharmacogenomics and its ability to be used effectively are the technologies that are available (1). This paper attempts to provide a review of some of the current technologies available for genotyping.

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Restriction fragment length polymorphism (RFLP) analysis

Restriction fragment length polymorphism (RFLP) analysis is one of the hallmark methods used to identify known mutations. This method is based on the ability of bacterial enzymes to recognize a specific 4–6-base-long sequence of DNA and cleave it, producing DNA fragments with blunt or sticky ends. However, the desired region of DNA containing the polymorphism must first be amplified by polymerase chain reaction (PCR). The amplified PCR product is then digested with an appropriate restriction endonuclease, separated by gel electrophoresis, and stained for visualization. The main limitation of this methodology is that the polymorphism must alter or generate a restriction site, resulting in a change in the DNA fragmentation (restriction digestion) pattern on the gel. RFLP analysis has been successfully used to genotype several key drug-metabolizing enzymes, including cytochrome P450 (*CYP*) *2D6* (2), *2C9*, and *2C19* (3). The main drawback of this technique is that it is labor-intensive, costly, and not suitable for large-scale clinical applications. RFLP can also be used with genomic DNA and Southern blotting techniques to provide information regarding genetic locus alterations, gene deletions and gene duplications. Chaves et al. have successfully used RFLP analysis of the low-density lipoprotein receptor locus to investigate the molecular genetics of familial hypercholesterolemia (4). RFLP and Southern blot analysis has also been used to distinguish *Brucella melitensis* strains from other *Brucella* species, indicating it may be useful for typing and diagnostic purposes as well (5).

Allele-specific amplification using real-time PCR

Recent developments in PCR equipment have made an impact on pharmacogenomic testing. The newer technologies now have the capability to amplify and detect product formation during the PCR reaction, which is commonly referred to as real-time PCR. This ability represents a major advantage and time saving feature, since it does not require post-PCR analysis (i.e., gel electrophoresis).

LightCycler®

One technology, the LightCycler® (Roche Molecular Systems, Pleasanton, CA, USA) offers the real-time detection of sequence-specific single nucleotide polymorphisms (SNPs). This method uses two fluo-

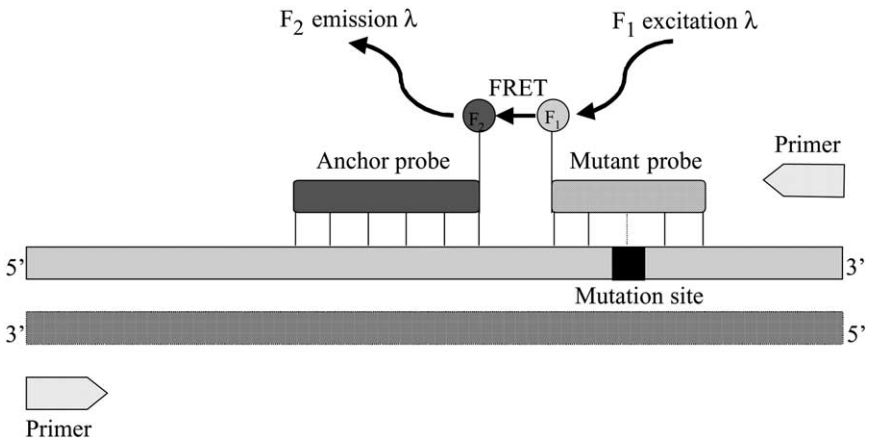

Figure 1 A schematic of a single-color one-point mutation experiment on the LightCycler®.

rescently labeled probes (an anchor probe and a mutation probe) that during amplification can detect specific single copy sequences in genomic DNA, or identify single-base mutations. Each probe is labeled with a different marker dye and hybridizes in a head-to-tail arrangement adjacent to one another on the target DNA (Figure 1). The two dyes can only interact when they are both bound to the target DNA and in close proximity. The mutation probe binds over the predicted site of the mutation and contains the donor molecule. The anchor probe contains an acceptor molecule and ultimately produces the fluorescent signal that is monitored. Both probes must bind to the DNA in close proximity so that fluorescent resonance energy transfer (FRET) can occur between the two probes. In order for FRET to occur, the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor. Software tools are available to aid in the design of the probes, making it relatively simple. Variant polymorphisms are identified in the amplified product by melting curve analysis. After the PCR amplification is complete, the temperature is lowered so that the probes are hybridized to the DNA. The temperature in the reaction chamber is then slowly increased while measuring the fluorescence at frequent intervals. Fluorescence will decrease with increasing temperature as the mutation probe is released and FRET ceases. If a SNP is present, the mutation probe has one base pair mismatched and will melt off at a lower temperature than if the SNP is absent. Melting curve analysis has been successfully applied by several groups to detect several *CYP2D6* polymorphisms [**3* and **4* (6); **3*, **4*, **6*, **7* and **8*, (7)]. The principle of discrimination between genotypes by melting curve analysis is also applied in the LightCycler® detection kits for factor V Leiden, prothrombin, apolipoprotein B, and apolipoprotein E. This technology has several benefits, including rapid real-time analysis and a closed system to prevent DNA contamination.

Invader®

A nuclease-based approach (Invader®) to detect known polymorphisms was developed by Third Wave Technologies (Madison, WI, USA) (8, 9). During the Invader® process, a proprietary enzyme (Cleavase®) recognizes the structure formed when the two specifically designed DNA probes bind to the target DNA. The two probes (a wild-type or variant probe and an upstream Invader® probe) are designed so that they hybridize to and partially overlap a known SNP site. The binding of the Invader® probe forces a small section (5′ end) of the overlapping wild-type or variant probe to remain non-hybridized (Figure 2). The overlapping flap is recognized by Cleavase®, which then cleaves and releases the small fragment of DNA (flap) (10, 11). The released fragment of DNA then undergoes a secondary reaction and binds to a 5′ fluorescently end-labeled probe (FRET™ cassette) forming another cleavage structure. The FRET™ cassette has a donor fluorophore that is quenched by an internal acceptor dye. However, when the cleavage structure formed by the FRET™ cassette and DNA flap is cut by Cleavase®, a fluorescent signal is detected. The Invader® system relies on this linear amplification of the signal rather than amplification of the target DNA. Each flap (cleavage product) can generate thousands of signals per hour, yielding millions of detectable signals per target. The major advantage of this technology is genotyping directly from genomic DNA without PCR amplification. However, this technology has limited multiplexing capabilities and cannot detect small insertions or deletions (12).

An application analysis for high-throughput analysis was demonstrated by Mein et al., where 384 individuals were genotyped for 36 SNPs and one insertion/deletion polymorphism (13). This study showed an average failure rate of 2.3% and an accuracy of 99.2% compared to results generated with established methods. Most of the failure was attributed to a PCR step. The major disadvantage is the need to assay each allele for a given SNP separately.

TaqMan™ DNA probes (real-time PCR)

Another technology (TaqMan™) takes advantage of the 5′ nuclease activity of *Taq* polymerase for allelic determination (14, 15). The desired region of DNA containing the SNP is amplified by PCR. However, two

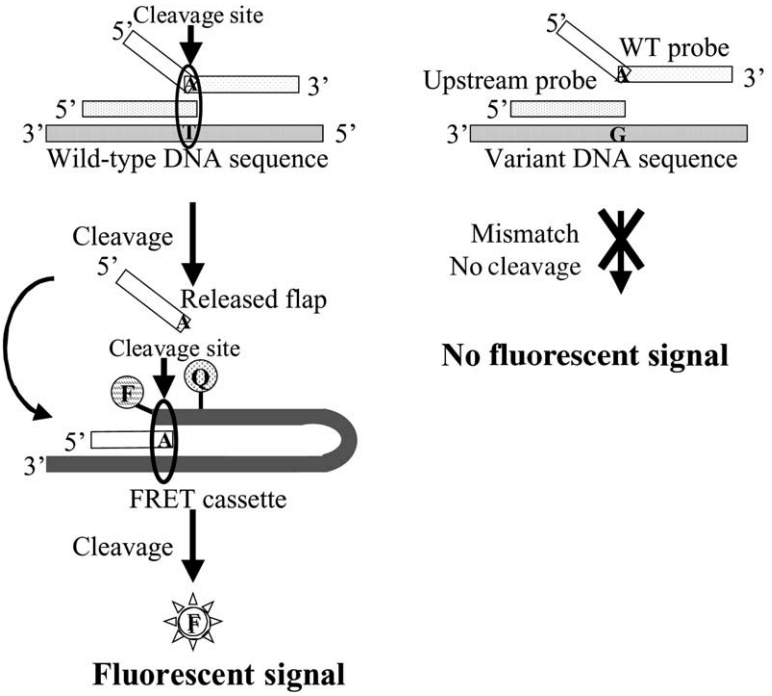

Figure 2 A schematic demonstrating the principles used in the Invader® assay.

TaqMan™ probes that differ only at the polymorphic site are also incorporated in the reaction. One probe is made complementary to the wild-type allele and the other probe to the variant allele. Each probe has a different covalently attached reporter dye and a common quencher dye on the 5′ and 3′ end, respectively. The probes bind to the polymorphic site of interest during the annealing phase of the PCR reaction. In the extension phase of PCR, the 5′ nuclease activity of *Taq* polymerase will cleave the 5′ reporter dye from the perfectly hybridized probe, resulting in increased fluorescence. Only the perfectly hybridized probe (wild-type or variant) will be cleaved by *Taq* polymerase, resulting in the real-time detection of PCR products (16). The ratio of the fluorescence from the two reporter dyes after the PCR reaction determines the genotype of the sample. The advantage of this methodology is that Applied Biosystems (Foster City, CA, USA) has ready-to-use, validated assays for genotyping common polymorphisms, providing both the reagents and instrumentation. The main disadvantages are that dual-labeled probes and PCR amplification are needed for genotyping, increasing the cost. However, this technology can easily be automated to increase throughput. Ranade et al. reported genotyping of more than 1600 individuals for two separate SNPs, with an error rate of less than 1/2000 (17).

Depolymerization assay (ReadIt™)

Promega Corporation (Madison, WI, USA) has created the ReadIt™ system that utilizes a proprietary polymerase, READase™, to catalyze depolymeration (pyrophosphorylation) of DNA. The ReadIt™ system can be used to analyze a wide variety of sequence variations, including insertions, deletions, and SNPs. This technique requires PCR amplification and depends on detection of light produced via the luciferase–lucifenin reaction. One basic aspect of the assay is that one of the PCR primers contains three phosphorothioate linkages at the 5′ end to protect one strand from nuclease degradation. Following the PCR amplification, T7 gene 6 exonuclease digests the unprotected strand in the PCR product to produce single-stranded templates. The single strand is allowed to hybridize to the interrogation probes. In the presence of correctly paired target DNA and probes, a depolymerization reaction occurs that is driven by mass action, resulting in the release of the high-energy deoxynucleotide triphosphate (dNTP) from the 3′ end of the DNA template. The READase™ kinase enzyme transfers the terminal phosphate from the dNTP onto ADP, forming ATP. Generation of ATP in the presence of luciferase results in light signal production that is proportional to the amount of ATP formed in the coupled reaction (Figure 3). The specificity of this system is provided by the sequence-specific hybridization of one or both oligonucleotide probes to the target DNA. The depolymerization reaction is contingent on the formation of a perfect homology at the 3′ end of the DNA sequence of interest. This technology can be partially automated for high-throughput analysis. Some applications of this technology are for *CYP2C9* (**2* and **3*) (18) and the identification of factor V Leiden polymorphism (19).

Tm Bioscience

Tm Bioscience (Toronto, Ontario, Canada) is a DNA-based diagnostics company that has developed sev-

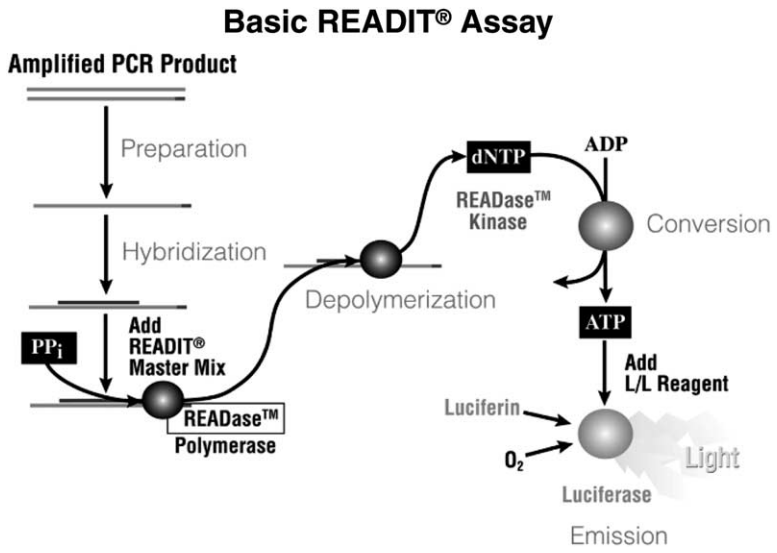

Figure 3 A schematic showing the principle of the ReadIt™ assay.

eral tests for genetic mutations related to coagulation, cystic fibrosis, and toxicology. The Tag-It™ mutation detection kit for *CYP2D6* simultaneously detects 12 variants and two gene rearrangements in a multiplex format (20). The Tag-It™ kit uses a multiplex PCR to amplify the target DNA of interest followed by allele-specific primer extension (ASPE). The method uses universally tagged allele-specific primers, of which the 3′ ends define the alleles, and a thermophilic DNA polymerase is used for primer extension and biotin-dCTP incorporation. Only the correctly hybridized primer(s) will be extended and will generate labeled product(s). Following ASPE, the products hybridize to bead-immobilized Tag compliments and a fluorescent reporter molecule (streptavidin-phycoerythrin) is used to indirectly detect incorporated biotin. The fluorescent signals generated for each bead population are read using the Luminex® xMap™ system and analysis is performed using the Tag-It™ software. Up to 50 SNPs can be genotyped using the Tag-It platform in one tube at a low cost. The Tag-It™ mutation detection kits offer an economical solution for laboratories requiring low-density multiplexing and high sample throughput. Assays are available for the detection of mutations in the *CFTR* gene, factor V Leiden, prothrombin, methyltetrahydrofolate reductase *(MTHFR)*, and *CYP2D6*. In addition, Tm Bioscience also has a microarray biochip (Tm100 Universal array) capable of combining any set of 100 single DNA tests and performing them simultaneously in a single reaction. The Tm100 universal microarray offers an accurate, fast, flexible, and economical platform for genetic analysis. The Tm100 is commercially available through Luminex. However, the Tm100 is also available for out-licensing on bead-based arrays, two-dimensional microarrays, and biosensors. Furthermore, the web-based design software (Tag-It™ Oligo design) simplifies the ordering of tagged gene-specific oligonucleotides. The software filters out any combinations that exhibit secondary structure or self-hybridizing characteristics.

EraGen

Two different platforms (Genecode and Multicode) are manufactured by EraGen (Madison, WI, USA) using an expanded genetic information system (AEGIS). EraGen has expanded the genetic alphabet with the development of eight new synthetic DNA bases. As a result, six more DNA base pairs are possible, in addition to the two traditional DNA base pairs found in nature (adenosine/thymine and guanine/cytosine). The new synthetic bases allow the inexpensive and rapid development of fully automated genetic diagnostic tests. AEGIS has already been used in FDA-approved commercial tests for managing HIV and hepatitis C infections in patients (21).

The Genecode™ 2.0 platform allows quantification, mixed population genotyping, infectious disease analysis, and gene expression analysis. The GeneCode platform is currently being used for the detection of severe acute respiratory syndrome (SARS). It offers single-copy detection, high specificity, excellent reproducibility, rapid results, and multiplexing capabilities. In addition, it is compatible with most real-time PCR instruments, has no post-PCR processing, and internal controls.

MultiCode is a cost-effective ultra-high-throughput multiplexed system used for SNP analysis or genetic predisposition testing (i.e., cystic fibrosis). The key to both platforms is that the concern of any cross-reactivity with natural DNA is eliminated due to the expanded genetic alphabet (AEGIS). Basically, the AEGIS coded primers extend the target specifically and a labeled AEGIS triphosphate is incorporated at a precise site, generating a product that can be captured at room temperature and quantitatively analyzed by fluorescence using a Luminex LabMAP instrument. The Luminex bead type is coupled to a specific EraCode that the AEGIS-coded extension primer recognizes. The main benefits of this method are the ability to rapidly (3–4 hours) and cost-effectively acquire multiple targets in a single reaction.

Denaturing high-performance liquid chromatography (dHPLC)

dHPLC is another technology that can distinguish between different DNA fragments in a heterogeneous mixture. The dHPLC is a high-performance liquid chromatography system that can be used for the separation, detection and collection of nucleic acid fragments. Separation of different DNA fragments is based on the binding affinity of the HPLC column that is directly related to the nucleic acid content of each fragment. Therefore, PCR products up to 1000 bp in length can be screened for SNPs at high accuracy when digested with restriction enzyme to create fragments smaller than 600 bp. The different fragments bind with different affinities and are eluted from the column at different times. This technology lends itself to SNP detection, oligo purification, quantification, fragment sizing, and DNA fingerprinting. The advantage of this technique is that it provides a rapid, cost-effective, and accurate method of identifying sequence variations (known or unknown) in PCR products (22). The typical run time for SNP detection is 10 minutes per sample. dHPLC is also very sensitive, since it is capable of detecting a 1-bp difference in a 750-bp fragment (23). dHPLC has been commercialized (WAVE® and Helix®) by two companies, Transgenomic Inc (San Jose, CA, USA) and Varian Inc (Palo Alto, CA, USA), and has been used for analyzing mutations in BRCA1 and BRCA2 (24).

Pyrosequencing™

Pyrosequencing (Pyrosequencing Inc, Uppsala, Sweden) is based on sequencing by synthesis, where the extension of complementary sequence is monitored rather than the termination. Like most other methods, PCR amplification is required and is used to generate biotinylated strands. The biotin incorporation is required to facilitate immobilization of the strand. This step is followed by the sequencing reaction. Addition of one of the four dNTPs in the presence of DNA polymerase, ATP sulfurylase, luciferase, and apyrase ensures signal production upon incorporation of added dNTP. Generation of the light signal is proportional to the amount of incorporated dNTP and is detected by a charge-coupled device (CCD) camera and seen as a peak in the Pyrogram™. Unincorporated dNTPs and generated ATP are degraded by apyrase before the addition of the next dNTP to turn the signal off. Each dNTP is added in a predetermined manner, one nucleotide at a time, to up to 30–40 bases or until the sequence information needed is attained (Figure 4). Pyrosequencing shows promise not only for clinical and research applications, but also in forensic medicine. This method provides rapid turnaround time and multiplexing capabilities. In addition, it also offers increasing discrimination power for mitochondrial DNA analysis by allowing sequencing of the coding region as well as the variable regions of the D-loop from mtDNA (25).

Mass spectrometry

Due to the great accuracy it provides in distinguishing small mass differences, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) techniques have been utilized for a variety of DNA analysis, including genotyping. One clinical application is the detection of the cystathionine β-synthase (*CBS*) gene deficiency that results in homocystinuria (26). MALDI-TOF-MS has also been used in detection of Toll-like receptor (TLR4) polymorphisms in patients to predict susceptibility to bacterial infection (27). Most of these analyses require a purification step prior to MS analysis, yet introducing a single charge (positive or negative) to the target can increase the sensitivity. One such application is the ''GOOD assay'' reported by Sauer et al. (28). The advantages of this system are the omission of the purification step and the ability to add all the reagents in a single reaction tube, followed by mass spectroscopy analysis. Like most other techniques, PCR amplification of the target DNA is required followed by the primer extension step. Since dNTPs from this step need to be removed prior to primer extension, either shrimp alkaline phosphatase or Tma 31 FS DNA polymerase, which have a preference for ddNTPs, are used. Introduction of the single charge is achieved in the primer extension step by the utilization of extension primers containing a charged tag that anneals immediately upstream of the SNP to be genotyped. Thermocycling in the presence of a specific set of α-S-dNTP and α-S-ddNTP results in allele-specific extension products containing phosphorothioate bridges. Following the extension reaction, the primers are digested by phosphodiesterase. However, the 3′ ends of the primers are not susceptible to the digestion since they contain phosphorothioate bridges. The alkylation reaction is optimized only to add methyl groups to the bridge thiolates. The sample is mixed with cyano-4-hydroxy-cinnamic acid methyl ester and applied to the target. In the range of 1000–2000 Da, MALDI-TOF-MS has a resolution power of 4 m/z. Since the smallest difference due to a SNP is 9 m/z, (A-T change) this technique provides the required detection sensitivity. An additional benefit of multiplexing is gained due to the requirement of different detection modes for positively and negatively tagged products. Also, the middle base of the extension primer harboring the amino-modification for the positive mode can be used as a mass tag location, introducing an alternative for multiplexing.

MALDI-TOF-MS combined with a chip as the target surface is presented by the SEQUENOM® (San Diego, CA, USA) MassARRAY™ system. This system is based on generations of allele-specific masses, through a primer extension reaction, that are further loaded onto a chip for detection. Biotinylated primers are

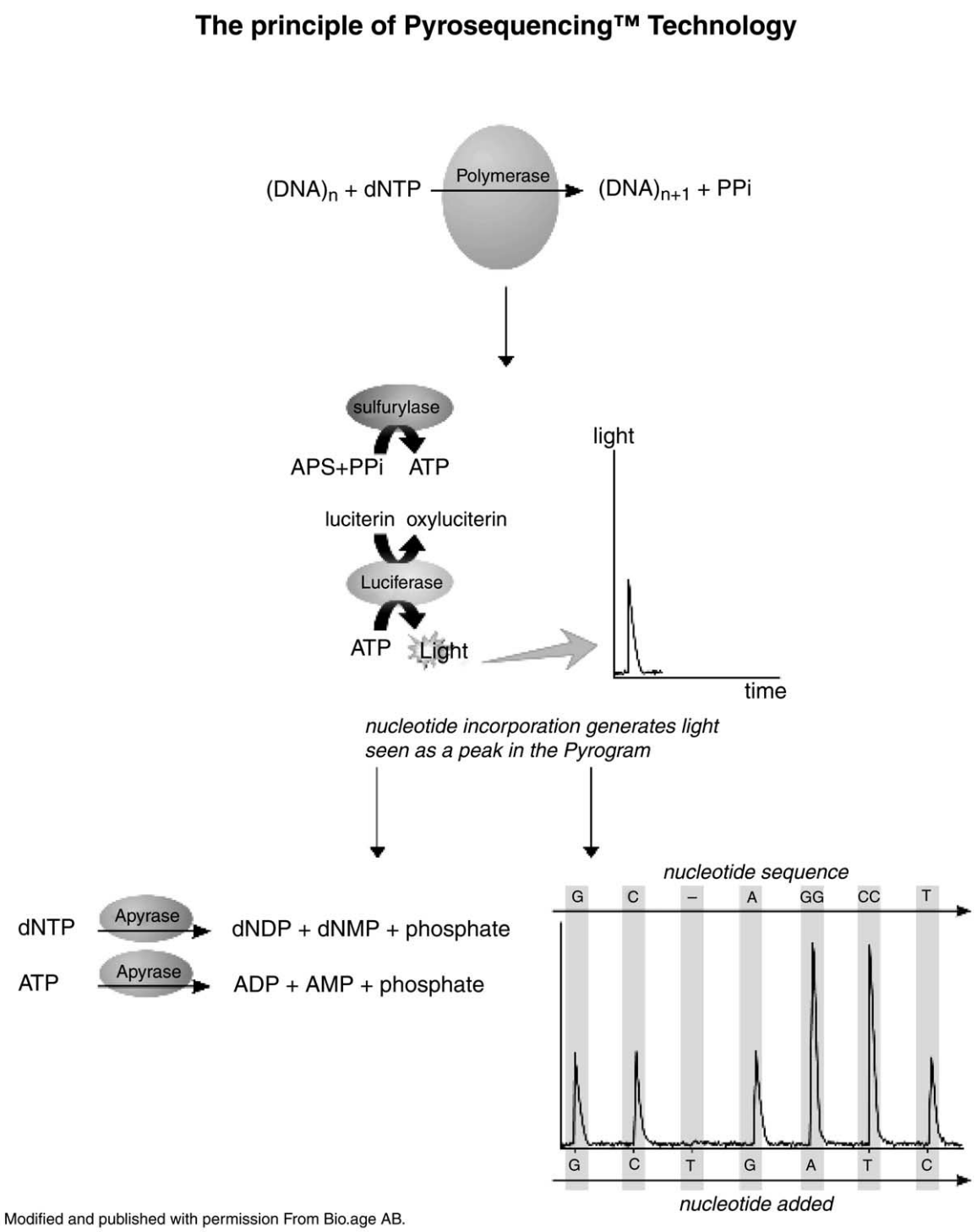

Figure 4 A schematic showing the principle of Pyrosequencing™.

used during the PCR amplification, followed by strand separation and an extension reaction over the SNP of interest. The extension reaction, controlled by the ddNTP concentrations, over the SNP of interest generates two products through the addition of two nucleotides to the specific MASSEXTEND™ primer. Following denaturation, the extension products are added to SpectroCHIPs™ for analysis by mass spectrophotometer using SpectroTYPER™ software.

The Survivor assay (29) is an alternate application of mass spectrophotometry for SNP detection. Electrospray ionization mass spectrophotometer (ESI-MS) is also used to detect single-nucleotide primer extensions (SNuPE).

Microarrays

Several high-density oligonucleotide arrays or DNA chips are being manufactured for elucidating genetic expression profiles along with SNP detection. DNA chips such as the AmpliChip CYP450 test by Roche

Molecular Systems Inc (Branchburg, NJ, USA) contain oligonucleotide probes that are anchored on glass supports. The AmpliChip CYP450 DNA chips can genotype 31 known polymorphisms in the *CYP2D6* gene, including gene duplication and deletion, as well as two mutations in the *CYP2C19* gene. Genomic DNA is first amplified by PCR in two separate reactions. The DNA amplicons are fragmented with DNase I and labeled at their 3′ termini with a Roche proprietary TdT labeling reagent and hybridized to the probe microarray using the GeneChip® 400 fluidics station. The microarray is subsequently washed and stained with a conjugated fluorescent dye and scanned by a GeneArray® scanner using a laser that excites the fluorescent label. The amount of light emitted is proportional to the bound target at each location on the probe microarray. Software analyzes the relative extent of probe hybridization to complementary mutant and wild-type targets on the microarray, and the corresponding genotypes are identified and displayed in the software report tool window.

An advantage of this DNA chip is the amount of data that is generated. This technology also allows for the simultaneous screening and detection of multiple mutations from several genes that have clinical relevance. Gonzalez and colleagues (30) have described a novel DNA probe array that simultaneously detects 204 human immunodeficiency virus type 1 antiretroviral resistance mutations. Currently, the expense of these DNA chips and the special equipment required for this genotyping technology are limiting factors for its widespread clinical application.

AutoGenomics INFINITI™ system

An automated approach for cost-effective genotyping has been released by AutoGenomics (Carlsbad, CA, USA). The self-contained, bench-top INFINITI™ system performs genomic and proteomic analysis in a continuous flow system (31). This enabling technology combines microarray and microfluidic methodologies with robotics, integrating the discrete processes of sample handling, reagent management, hybridization, and detection for DNA analysis. It utilizes a BioFilmChip™ microarray system for genetic mutational analysis of CFTR, factor V Leiden, prothrombin, and MTHFR, with other assays such as CYP2D6 to follow. The design of the system allows the adaptation of several methodologies, including hybridization assays, primer extension, competitive and sandwich immunoassays for performing mutational analysis of SNPs or short tandem repeats (STRs). The BioFilmChip™ microarray is available in two formats, one that is disease-specific and a second generic chip that enables researchers to anchor their own appropriate probes and develop assays. The INFINITI™ automated process can identify patient samples and query the laboratory information system for which assay to run, indicate what reagent modules or BioFilmChips to load, monitor the assay, and deliver the results in real time. It also has the flexibility to operate in a random access manner and can process up to 24 different microarray chips simultaneously on different patient samples.

Nanogen

Another technology that uses an electronic microarray is the Nanochip® system (Nanogen, San Diego, CA, USA). The Nanochip® system is comprised of three major subsystems: a Nanochip® loader for adding patient samples to the Nanochip® cartridge, a Nanochip® reader, and computer hardware/software for data analysis. The Nanochip® electronic microarray is a small silicon chip that contains 100 test sites laid out in a geometric grid. Nanogen's chip uses an electric current to charge individual test sites on the microarray. Since the majority of biological molecules such as DNA and RNA have positive and negative charges, the molecules can be electronically addressed to specific test sites. This process of electronic addressing can accelerate molecular binding to up to 1000-fold faster than traditional passive methods, as well as facilitate the concentration of the biotinylated sample. Once the biotinylated DNA samples are electronically addressed, complementary DNA reporter probes are hybridized, and any unbound or non-specifically bound DNA is removed. Stringency can be achieved using electronic, thermal or chemical techniques. The Nanochip® reader then scans the microchip using a two-laser system and measures the fluorescence. The software than can analyze the data and display the results using tables and/or histograms. This platform can be used for SNP analysis, short tandem repeats, and insertions or deletions. Currently, Nanogen offers several analyte-specific reagents that have been validated for CFTR, factor V Leiden/prothrombin, ASPA, hereditary hemochromatosis, and apolipoprotein E (apoE) mutations associated with Alzheimer's disease. One major advantage of the Nanochip® system is that it is an open platform, so customers can customize their own assays. An example application was reported by Moutereau et al. (32) who used the NanoChip® molecular biology workstation and developed a reasonably priced assay to detect the frequent mutations involved in familial Mediterranean fever.

Nanosphere

Another promising new platform (Verigene™) by Nanosphere (Chicago, IL, USA) uses nanotechnology-based nanoparticle probes, assays, and instruments for the detection of DNA, RNA, and/or protein. The Verigene™ platform offers unparalleled accuracy, speed, simplicity and versatility using a proprietary ClearRead™ technology. The technology revolves around complementary DNA sequences coupled to small (diameter ~13 nm) gold particles that specifically bind to target DNA sequences. The ClearRead assay involves hybridization of target DNA to both

nanoparticle probes and capture strands, followed by signal amplification with silver. The silver catalyzes on the gold nanoparticle, resulting in the enhancement of the signal by six orders of magnitude. As a result, this technology offers up to 10,000-fold greater sensitivity than standard methods and the ability to detect target DNA without amplification (PCR). Other advantages of the gold nanoparticle system include the ability to detect multiple targets in the same assay, low background noise, narrow temperature range for DNA denaturation, and the nanoparticle probes are non-toxic and stabile. Initially, Nanosphere offers SNP tests for hypercoagulation disorders (factor V Leiden/prothrombin) with future assays being developed for infectious diseases, cancer, and pharmacogenomics. An automated version of the Verigene ID system is also planned with integrated fluid processing for increased throughput and one-step processing.

Conclusion

Overall, a large number of genotyping technologies are available to detect known and unknown polymorphisms. The success and clinical application of pharmacogenomics depends on having simplistic, sensitive, rapid, and accurate techniques. Each of the methods discussed above has specific advantages and limitations. Currently, real-time PCR applications like those available for the LightCycler™ are among the simplest and fastest methods for mutation detection, since no post-PCR processing is required. In addition, assays like factor V Leiden and factor II are FDA approved. Pyrosequencing offers a distinct advantage, in that the DNA sequence surrounding the mutation is obtained. In general, sequencing has always been regarded as the gold standard for mutation detection. In the future, nanotechnologies like the Verigene™ platform from Nanosphere will become more important, since detection will be automated and not require amplification (PCR). Newer technologies that do not require PCR will avoid having to pay royalties and minimize the turnaround time and labor for genetic tests. Ultimately, each laboratory will have to choose the technology that best fits their physical and financial needs.

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